METHODS AND COMPOSITIONS FOR CONTROLLING DENTAL CARIES, AND RECOMBINANT SmaA POLYPEPTIDES USEFUL FOR SAME

REFERENCE TO RELATED APPLICATION

This application claims the benefit of United States application Serial No. 60/132,312 filed May 3, 1999, which is hereby incorporated herein by reference in its entirety.

BACKGROUND TO THE INVENTION

The present invention relates generally to the control of dental caries, and in particular to methods and compositions for controlling dental caries in hosts which involve the oral administration of polypeptides. The invention also relates to recombinant polypeptides having an amino acid sequence corresponding to that of SmaA, an amylase-binding adhesion from *Streptococcus mutans*, to isolated DNA sequences encoding such polypeptides, and to constructs and methods employing such DNA sequences.

As further background, over the past two decades the adhesive characteristics of bacteria and other prokaryotic and eukaryotic cells have attracted a great deal of attention, and their significance in microbial ecology and pathogenesis has become increasingly apparent (reviewed in K. Krogfelt, *Rev. Infect. Des.*, 13:721-735 (1991) and C.J. Smyth et al., FEMS Immunology and Medical Microbiology, 16:127-139 (1996)). Adhesion to the host surface is a necessary first step in the pathogenicity of microorganisms involved in the development of many diseases, including dental caries. Bacterial fimbriae-mediated interactions have been postulated as one mechanism by which oral bacteria adhere to the tooth surface (S. Fachon-Kalweit et al., *Infect. Immun.*, 48:617-624 (1985); B.L.

Elder et al., *Infect. Immun.*, 54:421-427 (1986); P.M. Fives-Taylor, *Infect. Immun.*, 55:123-128 (1987); and R.J. Gibbons et al., *Infect. Immun.*, 41:414-417 (1983)). Bacterial fimbriae are defined as small (100-300 nm) nonflagellar filamentous proteinaceous surface appendages that do not participate in the transfer of nucleic acids (L.O. Bakaletz et al., *Infect. Immun.*, 56:331-335 (1988)). The fimbriae-mediated adhesive properties of gram-negative bacteria have been extensively studied in medicine (e.g., *E. coli* infections; H. Schroten et al., *Infection*, 20:273-275 (1992)) and dentistry (e.g., *Porphyromonas gingivalis* and periodontal disease; T. Ogawa et al., *Immunol.*, 69:8-13 (1990)); however, relatively few fimbriae studies have been conducted on oral gram-positive microorganisms. Before a microorganism can cause disease, it must first find a portal of entry. The next step in this complex process is to attach to the host surface. Many human pathogens have developed through evolutionary mechanisms structures that aid in attachment. Several organisms such as *Neisseria gonorrhea* and *E. coli* have surface appendages that aid in adherence to the host tissues. These structures have been well described on other gram negative organisms (M.A. Lowe et al., *J. Bacteriol.*, 169:157-163 (1987)). The ultrastructure of gram negative fimbriae is composed of a protein known as pilin which winds around a central core. The fimbriae of the family *Enterobacteriaceae* have been extensively studied. It has been determined that there are two basic types of fimbriae: those which are mannose sensitivity (type 1) and those that are mannose resistant, although there are a number of other fimbrial types. The monosaccharide, D-mannose, has the ability to inhibit the attachment of the type 1 fimbriae (J.P. Duguid et al., *J. Pathol. Bacteriol.*, 75:519-520 (1958); J.P. Duguid et al., *J. Pathol. Bacteriol.*, 92:107-138 (1966); J.P. Duguid et al., *J. Med. Microbiol.*, 8:149-166 (1975)). *E. coli* contains type 1 fimbriae that have different serological properties between strains (J.E. Salit et al., *J. Exp. Med.*, 1468:1169-1179 (1977); reviewed in K. Krogfelt, *Rev. Infect. Dis.*, 13:721-735 (1991) and C.J. Smyth et al., FEMS Immunology and Medical Microbiology, 16:127-139 (1996)). In addition, the type 1 fimbriae from *E.*

coli do not cross react with type 1 fimbriae from *Salmonella typhimurium* (T.K. Korhonen et al., *J. Bacteriol.*, 144:800-805 (1980)). This fact provided an opportunity for a vaccine, because an effective vaccine should not cross react with other species. Cross reactivity across many species may lead to changes in the ecological balance of the microbial flora, and that may be detrimental to the host.

The appendages from the gram positive organisms have distinctly different structures than the gram negative bacteria. In particular, the gram negative fimbriae are composed of repeating subunits of one protein, while gram positive fimbriae contain several different proteins with well differentiated functions. Much controversy has arisen over surface appendage terminology in the oral streptococci. For example, J.C.G. Ottow, *Ann. Rev. Microbiol.*, 29:79-108 (1975), proposed that oral streptococci lack fimbriae but have a structurally distinct appendage known as a fibril. Fibrils are short structures usually less than 200 nm but up to 400 nm. These structures are peritrichous and appear in clumps, therefore, making width measurements very difficult (P.S. Handley, *Biofouling.*, 2:239-264 (1990)). These fibrils are found in various sizes on a particular bacterial cell. For example, *Streptococcus salivarius*, a bacterium that inhabits the dorsum of the tongue, has two distinct fibrils of differing lengths. The first, AgB, the longer fibril, serves to attach to *Veillonella parvula*, and the second, AgC, binds to host tissues (Weerkamp and Jacobs, 1982). It is apparent that these fibrils are composed of separate glycoproteins with distinct structural and functional characteristics. However, the terminology in the field is unclear as other laboratories working with different oral streptococci, report the presence of fimbriae.

Studies conducted on *Streptococcus parasanguis*, one of the primary colonizers of dental plaque (L. Oligino et al., *Infect. Immun.*, 61:1016-1022 (1993)), have demonstrated that attachment to saliva-coated hydroxyapatite (HA) is mediated by a 36 kDa adhesin protein (FimA) which

is a component of the bacterial fimbriae, and is able to displace bound cells (S. Fachon-Kalweit et al., *Infect. Immun.*, 48:617-624 (1985); L. Oligino et al., *Infect. Immun.*, 61:1016-1022 (1993)). It has also been concluded that *S. parasangui* fimbriae are essential for the microorganism to attach, since wild-type fimbriated cells bind well to saliva-coated HA in an *in vitro* model, whereas afimbriated mutants do not (P.M. Fives-Taylor et al., *Infect. Immun.*, 47:752-759 (1985)). J. Fontana et al., *Clin. Diagn. Lab. Immunol.*, 2:719-725 (1995) demonstrated that the surface of *S. mutans* cells exhibits the presence of a fuzzy coat or fringe of constant width and density around the bacterial cell from which short fimbriae-like structures (100-200 nm) protrude. This type of surface fimbrilar structure has been described previously on a majority of *Streptococcus sanguis* biotype I and II strains, and is referred to as a peritrichous fibrilar structure. Other than the *S. sanguis* family (comprised of *S. sanguis*, *S. gordonii* and *S. parasanguis*), very little is known about the biochemical nature of gram-positive bacteria fimbriae. Part of the difficulty has arisen from complications in obtaining purified preparations because of the extremely hydrophobic nature of the fimbriae (P.M. Fives-Taylor et al., *Infect. Immun.*, 55:123-128 (1987)). In addition, methods that involve dissociation and depolymerization of fimbriae from gram-negative organisms are generally ineffective with gram-positive fimbriae.

A group of streptococcal genes encoding related FimA-proteins that function as oral adhesins has been identified and in some cases has been sequenced. These include FimA from *S. parasanguis* (J.C. Fenno et al., *Infect. Immun.*, 57:3527-3533 (1989)), SsaB from *S. sanguis* (N. Ganeshkumar et al., *Infect. Immun.*, 59:1093-1099 (1991)), ScaA from *Streptococcus gordonii* (P.I. Kolenbrander et al., *Infect. Immun.*, 62:4469-4480 (1994)) and PsaA from *Streptococcus pneumoniae* (J.S. Sampson et al., *Infect. Immun.*, 62:319-324 (1994)). Several of these reported streptococcal genes are conserved across species lines; SsaB shares 87% amino acid sequence identity with FimA (N. Ganeshkumar et al., *Infect.*

Immun., 59:1093-1099 (1991)). However, FimA was not present in *Streptococcus sobrinus*, suggesting that FimA was primarily present in the sanguis family (J.C. Fenno et al., *Mol. Microbiol.*, 15:849-863 (1995)). In general, gram-positive fimbriae have proved to be extremely difficult to dissociate into subunits, unlike fimbriae from gram-negative bacteria which traditionally have been reported to contain one protein composed of many identical subunits. However, *S. parasanguis* fimbriae have been demonstrated to contain peptides of 200, 160, 55 and 36 (FimA) kDa recognizable by polyclonal antisera. The 36 kDa FimA peptide was localized on the tip of the fimbriae by transmission electron microscopy, suggesting that the fimbriae are highly organized by having the adhesin located on the extreme end of the fimbriae making it accessible for binding to its ligand. In addition, insertional and deletional FimA mutants of the *S. parasanguis* wildtype strain were not able to induce endocarditis, whereas the wildtype strain was successful in induction of endocarditis (D. Burnette-Curley, *Infect. Immun.*, 63:4669-4674 (1995)). These results firmly establish the importance of FimA in *S. parasanguis*-induced endocarditis.

The structural genes carrying the *S. parasanguis* fimbriae are carried on a 6 kb fragment. The gene encoding for the 36 kDa FimA is located on a 0.9 kb fragment and is highly conserved in a sanguis streptococci (J.C. Fenno et al., *Mol. Microbiol.*, 15:849-863 (1995)). The carboxyl-terminal region of the protein does not have a hydrophobic membrane anchor sequence as found in other gram positive organisms (J.C. Fenno et al., *Infect. Immun.*, 57:3527-3533 (1989)), however it does contain the consensus prolipoprotein cleavage site reported as the periplasmic binding proteins of transport systems in gram positive bacteria (J.C. Fenno et al., *Mol. Microbiol.*, 15:849-863 (1995)). The FimA protein gene is expressed in both orientations which indicates that the promoter is present on the 6 kb fragment (P.M. Fives-Taylor et al., *Infect. Immun.*, 55:123-128 (1987)). The nucleotide sequence upstream of FimA contains 2 open reading frames (ORF5 and ORF1) which are homologous to ATP-

binding cassette membrane transport proteins (J.C. Fenno et al., *Mol. Microbiol.*, 15:849-863 (1995)). The ORF5 product is a 29 kDa membrane-associated protein and mutagenesis of ORF5 results in increased resistance to aminopterin, while the ORF1 product is a 31 kDa membrane protein. ORF3, encoding for a 20 kDa protein, is located downstream of FimA and is homologous to a protein located close to SsaB from *S. sanguis*. ORF5, ORF1, FimA and ORF3 are located as a polycistronic message, however, FimA mutants produce fimbriae, demonstrating that FimA is not the structural subunit.

Much work has also been conducted on the characterization of *A. naeslundii* (formerly *A. viscosus*) fimbriae. *A. naeslundii* has 2 types of fimbriae. Type 1 fimbriae mediates attachment of the organism to saliva-coated HA (M.K. Yeung et al., *J. Bacteriol.*, 169:1678-1683 (1987)), while type 2 fimbriae are associated with lectin activity (S.E. Mergenhagen et al., *Rev. Infect. Dis.*, 9:S467-474 (1987)). The type 1 subunit is a 65 kDa protein and the type 2 subunit is a 59 kDa protein (M.K. Yeung et al., *J. Bacteriol.*, 170:3803-3809 (1988)). The type 1 subunit (FimP) contains a carboxy-terminal membrane-spanning region (M.K. Yeung et al., *J. Bacteriol.*, 172:2462-2468 (1990)). In addition, there was significant amino acid homology (34%) between the 2 types of fimbriae. FimP was detected in approximately half of the *Actinomyces* strains examined, indicating a large degree of conservation across strains (M.K. Yeung, *Infect. Immun.*, 60:1047-1054 (1992)). FimP is flanked by 6 ORF, 3 are located 5' and 3 are 3' of FimP. ORF1 encodes for a protein of approximately 39 kDa and has significant nucleotide homology with both FimP and the type 2 fimbrial subunit. Insertional mutagenesis of ORF1 and ORF2 provided mutants that did not express type 1 fimbriae or FimP, whereas ORF3 or ORF4 mutants expressed only FimP. None of these mutants had binding activity for the proline-rich polypeptides (PRP) that type fimbriae bind to. ORF6 mutants had normal fimbrial structure and binding activity (M.K. Yeung et al., *Infect. Immun.*, 65:2629-2639 (1997)).

Relatively little work has been done on fimbrial structures of *S. mutans* and very few studies have examined purified fimbrial components. Ericson and colleagues in 1987 (D. Ericson et al., *J. Bacteriol.*, 169:2507-2515 (1987)) published a paper describing a "fuzzy coat" that surrounds the *S. mutans* cell. The fuzzy coat is approximately 50-100 nm wide with protrusions radiating through the halo at each pole. Ericson and colleagues also determined the structures to be heat stable, sonciation sensitive, and protease sensitive. They suggested a distinct possibility that these appendages serve as attachment structures with the receptor being salivary pellicle.

In light of this background there remain needs for methods and compositions for inhibiting the development of dental caries, and to substances useful therein. The present invention addresses these needs.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides methods for controlling dental caries in the oral cavity of a host. The methods comprise administering to the oral cavity of the host an effective caries inhibiting amount of a purified SmaA protein or an amylase-binding polypeptide fragment thereof. In such methods, the protein or fragment can be purified from natural sources or recombinantly-produced. Oral administration is desirably accomplished using an oral composition including the protein or fragment and an orally acceptable excipient. The oral composition may be, for example, an aqueous mouthwash or rinse composition, a dentifrice composition, or a chewing gum. Periodic application of the SmaA protein or fragment, for example at least once daily, will be preferred.

Another aspect of the invention concerns a method for controlling dental caries in a host, which comprises periodically administering to the oral cavity of the host a non-immunogenic peptide which competitively blocks adhesion of caries-causing bacteria, especially *S. mutans*, to the tooth surface. Administration of such non-immunogenic peptides can be used to competitively inhibit bacterial adhesion, while avoiding implicating the immune system of the host.

Another embodiment of the invention provides an oral composition for periodic topical application to block the binding of *S. mutans* to the salivary pellicle, comprising a substance such as a purified SmaA protein or an amylase-binding polypeptide fragment thereof which blocks attachment of *S. mutans* to the salivary pellicle, and an orally acceptable excipient.

The present invention also provides a recombinant polypeptide having an amino acid sequence corresponding to the amino acid sequence of SmaA or an active fragment thereof preferably having a molecular weight of at least about 0.5 kd.

In still another aspect, the invention provides an isolated DNA sequence encoding (i) SmaA protein or (ii) a polypeptide fragment of SmaA protein preferably having a molecular weight of at least about 0.5 kd. Also provided is a vector comprising such a DNA sequence in operable association with a promoter. As well, host cells having introduced DNA including such a DNA sequence in operable association with a promoter are provided, and can for example be cultured to produce SmaA or a fragment thereof.

The present invention provides novel methods and compositions for controlling dental caries in a host animal, including a human. The invention also provides novel isolated DNA sequences encoding SmaA polypeptides, constructs including such DNA sequences, and recombinant SmaA polypeptides. Additional objects, features and advantages of the invention will be apparent from the following description.

DESCRIPTION OF THE FIGURES

Figure 1 shows binding of *S. mutans* fimbriae to whole saliva-coated ELISA plates. The ability of *S. mutans* fimbriae (0.33 - 33.00 μg/ml) to bind to saliva (undiluted and 1:2 and 1:10 dilutions) was determined by ELISA. The negative controls were wells that did not contain fimbriae. The ELISA absorbances (mean ± SEM) represent a relative measurement of binding between fimbriae and saliva. ND = not determined.

Figure 2 shows representative immunoblot of whole saliva from seven different subjects. Blots were probed with fimbriae from *S. mutans* A32-2, followed by rat antibody to enriched fimbriae of *S. mutans* A32-2 and alkaline phosphatase-labeled goat antibody to rat IgG. Whole salivas from seven subjects (lanes 1-7) are shown. Arrow on right indicates molecular weight of the major salivary component (52 kDa) that bound fimbriae.

Figure 3 shows representative coomassie brilliant blue and silver dual stained SDS-PAGE gel of purified salivary protein. Purified salivary protein was collected using preparative gel electrophoresis and analyzed using SDS-PAGE. The arrow on the right indicates the molecular weight of the isolated salivary component (52 kDa).

Figure 4 shows *S. mutans* A32-2 fimbriae binding to salivary proteins. ELISA plate wells were coated with the purified salivary protein (65.0 μg/ml), whole saliva (diluted 1:10) and amylase (10.0 μg/ml). After blocking with 1% BSA, the *S. mutans* A32-2 fimbriae preparation (33.0 μg/ml) was incubated with the various salivary proteins. The controls (open bars) did not include fimbriae. The ELISA absorbances (mean ± SEM) represent a relative measurement of binding between fimbriae and salivary components.

Figure 5 shows neutralization of *S. mutans* fimbriae binding to saliva-coated surfaces by a purified salivary protein. The *S. mutans* A32-2 fimbriae preparation (33.0 µg/ml) was incubated with varying concentrations (0.5-65.0 µg/ml) of the purified salivary protein and assayed for binding to saliva.

Figure 6 provides a representative anti-amylase immunoblot. Purified salivary protein (65.0 µg/ml), undiluted whole saliva and human α-amylase (10.0 µg/ml) probed with rabbit antibody to human α-amylase. Human whole saliva (lane 1); human α-amylase (lane 2); and purified salivary protein (lane 3) are shown. The arrow on the right indicates a molecular weight of 52 kDa.

Figure 7 illustrates a plaque lift from repropagated positive plaque probed with rat anti-65 kDa fimbrial protein antibody and alkaline phosphatase-labeled anti-rat IgG, as described in Example 2 below. Positive spots indicate plaques from λ transfected *E. coli* cells expressing 65 kDa fimbrial protein. Every plaque produced a positive spot. Control plates with λ plaques without *S. mutans* insert did not express fimbrial protein and plaque lifts had no spots.

Figure 8 shows the results of an agarose gel electrophoresis of 9.5 kb *Sal*I fragment from λ DNA carrying *S. mutans* fragment screened with anti-65 kDa antisera, as described in Example 2 below. Lane 1 (l-r)-1 kb DNA ladder; lane 2-undigested λ DNA; lane 3-*EcoR*I digest; lane 4-*Sau3*A digest indicating complete digestion; lane 5-*Sma*I digest indicated 6.1 and 3.5 kb fragments; lane 6-*Hind*III digest indicating 5.0 and 4.5 kb fragments; and lane 7-*Sst*I digest.

Figure 9 shows a slotblot of saliva (Sal) and amylase (Am) probed with λ phage lysate (Ph) or crude fimbriae (Fim) followed by rat anti-

fimbriae antibody (FimAb) and anti-rat IgG reagent, as described in Example 2 below. All slots were blocked with 1% BSA, and rat anti-fimbriae antibody and alkaline phosphatase labeled anti-rat IgG antibody and AP substrate were added. Positive slots indicate presence of λ lysate or fimbriae binding to saliva or amylase. Column (left to right) 1 contains saline in place of saliva or amylase as negative control; column 2 contains saliva (1:10 dilution; rows A and B [top to bottom] and 1:20; rows C-D) and amylase (50 µg/ml; rows E-F and 25 µg/ml; rows G-H); columns 3 and 4 contain amylase at 50 and 25 µg/ml; columns 5 and 6 contain saliva at 1:20 (Sal-20) and 1:10 (Sal-10)dilutions. Rows A and B (columns 3-6) were probed with crude fimbriae (Fim; 10 µg/ml); rows C-D, E-F and G-H (columns 3-6) were probed with λ phage lysate (1:1000 [Ph-3], 1:10,000 [Ph-4] and 1:100,000 [Ph-5] dilutions, respectively), while columns 1-2 were incubated with saline in place of λ lysate and fimbriae bind to saliva and amylase.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

For the purpose of promoting an understanding of the principles of the invention, reference will now be made to certain preferred embodiments thereof and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations, further modifications and applications of the principles of the invention as described herein being contemplated as would normally occur to one skilled in the art to which the invention relates.

As disclosed above, the present invention provides methods and compositions for controlling dental caries in a host. As well, the invention provides novel recombinant protein SmaA and recombinant polypeptide fragments thereof, and isolated DNA sequences, vectors, and host cells suitable for the production of such recombinant proteins and fragments.

The purified SmaA protein involved in the present invention is an adhesion from fimbriae of *S. mutans* which mediates attachment of the bacteria to the salivary pellicle, believed to be *via* binding to the 52 kd salivary protein, amylase. The mature SmaA protein has a molecular weight of about 65 kilodaltons (kd) as measured on a reducing polyacrylamide gel, exhibits the ability to bind amylase, and is the major immunodominant fimbrial protein of *S. mutans*. The 65 kd band is not present when S. *mutans* fimbrial preparations are electrophoresed on a non-reducing polyacrylamide gel, and it is therefore believed that the 65 kd SmaA protein may be a subunit of a larger disulfide-bonded protein. Additional information concerning the SmaA protein and its uses can be found in Ray et al, Interactions of *Streptococcus mutans* Fimbrial-Associated Surface Proteins with Salivary Components, *Clin. Diag. Lab. Immunol.*, May 1999, 6(3) pp. 400-4, and M. Fontana et al., Intranasal

Immunization Against Dental Caries with *Streptococcus mutans* Enriched Fimbrial Preparation, *Clin. Diag. Lab. Immunol.*, May 1999, 6(3) pp. 405-9.

The applicant has also obtained amino acid sequence data believed to be from a sequence at or near the N-terminal portion of the protein, which is believed be EEQSGGT (SEQ. I.D. NO. 1) or YLMKGGT (SEQ. I.D. NO. 2). Additional sequence data show that the SmaA protein includes amino acid sequences of MSSQAKANNIP (SEQ. I.D. NO. 3) and MQRPTEFXEDK (SEQ. I.D. NO. 4), which are expected to be internal sequences in the protein. These sequences exhibit substantial identity to sequences within a 40 kd S. *mutans* cell wall protein precursor (strain OMZ15, serotype f) reported by J.A. Ogier et al., *Infection and Immunity*, May 1991, pp. 1620-1626 (see SEQ. I.D. NO. 6, and genbank accession no. A60328). The coding nucleotide sequence for such protein is shown in SEQ. I.D. NO. 5, from nucleotides 816 to 1820. Sequences similar to those shown in SEQ. I.D. NO. 6 and SEQ. I.D. NO. 5 are expected within the 65 kd SmaA protein and the DNA which encodes it, respectively. Thus, preferred aspects of the present invention involve uses as described herein of SmaA proteins or fragments thereof (desirably at least 0.5 kd) which have an amino acid sequence having at least about 70% identity to an amino acid sequence of SEQ. I.D. NO. 6, more preferably at least about 80%. Other preferred aspects of the invention involve uses as described herein of nucleic acid molecules which have a nucleotide sequence encoding a SmaA protein or fragment thereof, which nucleotide sequence has at least about 70% identity to a nucleotide sequence of SEQ. I.D. NO. 5 from nucleotide 816 to 1820, more preferably at least about 80% identity. Such nucleotides sequences preferably code for a polypeptide of at least about 0.5 kd.

Percent identity, as used herein, is intended to mean percent identity as determined by comparing sequence information using the advanced BLAST computer program, version 2.0.8, available from the National

Institutes of Health, USA. The BLAST program is based on the alignment method of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 87:2264-68 (1990) and as discussed in Altschul, et al., *J. Mol. Biol.* 215:403-10 (1990); Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-7 (1993); and Altschul et al. (1997) *Nucleic Acids Res.* 25:3389-3402. Briefly, the BLAST program defines identity as the number of identical aligned symbols (i.e., nucleotides or amino acids), divided by the total number of symbols in the shorter of the two sequences. The program may be used to determine percent identity over the entire length of the proteins being compared. Preferred default parameters for the BLAST program, blastp, include: (1) description of 500; (2) Expect value of 10; (3) Karlin-Altschul parameter λ = 0.270; (4) Karlin-Altschul parameter K = 0.0470; (5) gap penalties: Existence 11, Extension 1; (6) H value = $4.94e^{-324}$; (6) scores for matched and mismatched amino acids found in the BLOSUM62 matrix as described in Henikoff, S. and Henikoff, J.G., *Proc. Natl. Acad. Sci. USA* 89:10915-10919 (1992); Pearson, W.R., *Prot. Sci.* 4:1145-1160 (1995); and Henikoff, S. and Henikoff, J.G., *Proteins* 17:49-61 (1993). The program also uses an SEG filter to mask-off segments of the query sequence as determined by the SEG program of Wootton and Federhen *Computers and Chemistry* 17:149-163, (1993).

The applicant has cloned a 9.5 kb DNA insert from *S. mutans* which encodes the SmaA protein into the *BamH*I site of a λ gt11 universal expression vector. This vector has been used to transfect *E. coli*, which has been demonstrated to express the recombinant SmaA protein. Such recombinant protein and/or fragments thereof will be useful in a variety of applications, including for example in the implementation of vaccines against dental caries, in methods for competitive inhibition of *S. mutans* binding to oral surfaces such as teeth, and in assays involving amylase binding.

Additional polynucleotides encoding SmaA proteins may be obtained for example by generating DNA probes utilizing the sequences disclosed herein, and screening appropriate *S. mutans* DNA libraries using techniques within the purview of those practiced in this area.

SmaA proteins or fragments thereof can be used in active immunization protocols and/or to raise antibodies which may be used, for example, in passive immunization protocols to inhibit caries formation in mammals and other animals. For example, the isolated 65 kd SmaA protein has been used to rat antibodies to the protein, which have proven to react with all 20 strains of S. *mutans* tested to date, and to show lower or no significant reactivity to other oral bacteria.

In accordance with methods of the invention, the SmaA protein, or an active peptide derived from this protein, may be administered in an oral composition such as a dentifrice (e.g. toothpaste), a mouthwash, a chewing gum, or the like. Various conventional excipients and additives for such oral compositions, and their relative amounts, are well known. These are reviewed for example in U.S. Patent No. 5,500,206, portions of which are summarized and incorporated in the discussions below for ease of reference and in order to facilitate a description of certain components of oral compositions of the present invention.

In general, the oral compositions of the invention include an effective amount of the SmaA protein or an active fragment thereof in a suitable oral carrier. The SmaA protein or active fragment will typically be included at a level of about 0.01% to about 5% by weight of the oral composition, more preferably about 0.1% to about 2% by weight, and most preferably about 0.2 to about 1% by weight.

The oral carrier employed may comprise, for example, a solid or liquid filler, and diluents suitable for use in oral compositions intended for

contact with the oral tissues of humans and lower animals. Such compositions include for example mouthwashes, mouth rinses, mouth sprays, dental treatment solutions, toothpastes, dental gels, tooth powders, prophylaxis pastes, lozenges, chewing gums and the like. Dentifrices, mouthwashes and chewing gums represent preferred compositions.

The topical, oral carriers of the present invention comprise components typically used in such compositions which are well known to a skilled practitioner. Such components include, but are not limited to, anticaries agents, antiplaque agents, anticalculus agents, dental abrasives, surfactants, flavoring agents, sweetening agents, binders, humectants, thickening agents, buffering agents, preservatives, coloring agents and pigments, ethanol and water.

Water may be used in the topical, oral carriers of the compositions of the present invention, and when used will usually be of low ion content and free of organic impurities. Water may comprise from about 2% to about 99%, more typically from about 20% to about 95% of the compositions of the present invention. When in the form of toothpaste, the compositions preferably comprise from about 2% to about 45%, more preferably from about 30% to about 40%, water, while mouthwashes comprise preferably from about 45% to about 95%, more preferably from about 75% to about 90%, water.

A variety of dental abrasives will be useful in the topical, oral carriers of the compositions of the present invention. The material selected must be one which is compatible with the composition of interest and does not excessively abrade dentine. These include, for example, silicas, including gels and precipitates, calcium carbonate, dicalcium orthophosphate dihydrate, calcium pyrophosphate, tricalcium phosphate, calcium polymetaphosphate, insoluble sodium polymetaphosphate, hydrated alumina, and resinous abrasive materials such as particulate condensation

products of urea and formaldehyde, and other materials such as those disclosed by Cooley et al. in U.S. Pat. No. 3,070,510, issued Dec. 25, 1962. Mixtures of abrasives may also be used.

The silica abrasive polishing materials useful herein, as well as the other abrasives, generally have an average particle size ranging between about 0.1 and 30 microns, preferably between about 5 and 15 microns. The silica abrasive can be precipitated silica or silica gels such as the silica xerogels described in U.S. Pat. No. 3,538,230, issued Mar. 2, 1970 to Pader et al., and in U.S. Pat. No. 3,862,307, issued Jun. 21, 1975 to DiGiulio. Preferred are the silica xerogels marketed under the tradename Syloid.RTM. by the W.R. Grace & Company, Davidson Chemical Division. Preferred precipitated silica materials include those marketed by the J. M. Huber Corporation under the tradename, Zeodent.RTM., particularly the silica carrying the designation Zeodent 119.RTM.. These silica abrasives are described in U.S. Pat. No. 4,340,583, Wason, issued Jul. 20, 1982, incorporated herein by reference. Other suitable abrasives include alumina and the insoluble metaphosphates such as insoluble sodium metaphosphate (IMP).

Mixtures of abrasives may be used. The total amount of abrasive in the dentifrice embodiments of this invention can range from about 6% to about 70%, preferably from about 15% to about 50%, when the dentifrice is a toothpaste. Higher levels, as high as 90%, may be used if the composition is a tooth powder.

Flavoring agents can also be added to the oral compositions of the present invention. Suitable flavoring agents include menthol, oil of wintergreen, oil of peppermint, oil of spearmint, oil of sassafras, and oil of clove. Flavoring agents are generally included in the subject compositions in amounts of from about 0% to about 3%, preferably from about 0.04% to

about 2% by weight.

Coloring agents may be added to compositions of the present invention to improve appearance. If present, coloring agents typically are included at levels of from about 0.001% to about 0.5% by weight.

Sweetening agents may also be used in the compositions of the present invention. Sweetening agents which can be used include aspartame, acesulfame, saccharin salts, dextrose, levulose thaumatin, D-tryptophan, dihydrochalcones, and cyclamate salts. Saccharin salts are preferred. Sweetening agents are generally used in the subject compositions in amounts of from about 0% to about 6%, preferably from about 0.005% to about 5% by weight.

Oral compositions can also contain a surfactant. Suitable surfactants are those which are reasonably stable and form suds throughout a wide pH range, including nonsoap anionic, nonionic, cationic, zwitterionic and amphoteric organic synthetic detergents, and compatible mixtures thereof. Many of these suitable surfactants are disclosed in U.S. Pat. No. 4,051,234, issued to Gieske et al. on Sep. 27, 1977, and in U.S. Pat. No. 3,959,458 issued to Agricola, Briner, Granger and Widder on May 25, 1976. Surfactants are typically present in compositions of the present invention at a level of from 0% to about 10%, preferably from about 0.2% to about 4% by weight. Surfactants may also be used as solubilizing agents to help retain sparingly soluble components, e.g., some flavoring agents, in solutions. Surfactants suitable for this purpose include polysorbates and poloxamers.

In preparing oral compositions of the present invention, it is often desirable to add binders and/or thickening agents, particularly to toothpaste compositions. Preferred binders and thickening agents include for example, carboxyvinyl polymers, polysaccharide gums such as xanthan gum,

carrageenan, hydroxyethyl cellulose and water soluble salts of cellulose ethers such as sodium carboxymethyl cellulose and sodium carboxymethyl hydroxyethyl cellulose. Natural gums such as gum karaya, gum arabic, and gum tragacanth can also be used. Colloidal magnesium aluminum silicate or finely divided silica can be used as part of the thickening agent to further improve texture. These binders and thickening agents are generally present in the compositions of the present invention in amounts of from about 0% to about 6%, preferably from about 0.1% to about 5% by weight.

Another optional component of the oral carriers of the compositions of the present invention is a humectant. The humectant serves to keep toothpaste compositions from hardening upon exposure to air, and to give mouthwash and toothpaste compositions a moist feel to the mouth. Certain humectants can also impart desirable sweetness of flavor to mouthwash and toothpaste compositions. The humectant, on a pure humectant basis, generally comprises from about 0% to about 70%, preferably from about 2% to about 55%, by weight of the compositions herein. Suitable humectants for use in compositions of the present invention include edible polyhydric alcohols such as glycerin, sorbitol, xylitol, polyethylene glycol, and propylene glycol, especially sorbitol and glycerin.

Opacifiers may also be used in toothpastes of the present invention to render the toothpaste opaque. Suitable opacifiers include titanium dioxide and some abrasives including, for example, magnesium aluminum silicate. Opacifiers generally comprise from about 0% to about 4%, preferably from about 0.5% to about 3% by weight of the compositions herein.

Other optional components of the compositions of the present invention are preservatives. The preservatives prevent microbial growth in the compositions. Suitable preservatives include methylparaben, propylparaben, benzoates and ethanol. If the preservative is ethanol, it

generally comprises from 0% to about 35% by weight, preferably from about 5% to about 15%, of the compositions herein. Other preservatives generally comprise from about 0% to about 5% by weight, preferably from about 0.1% to about 2%, of the compositions herein.

Antimicrobial, antiplaque agents can also optionally be present in the oral compositions of the present invention, on the condition that they are compatible with the SmaA protein or fragment. Such agents may include, but are not limited to, triclosan, 2,4,4'-trichloro-2'-hydroxydiphenyl ether, as described The Merck Index, 11th Ed. (1989), p. 1520 (entry No. 9573); in U.S. Pat. No. 3,506,720; and in Eur. Pat. Appl. No. 0,251,591 of Beecham Group, PLC, published Jan. 7, 1988, chlorhexidine, (Merck Index, No. 2090), alexidine (Merck Index, No. 222); hexetidine (Merck Index, No. 4624); sanguinarine (Merck Index, No. 8320); benzalkonium chloride (Merck Index, No. 1066); salicylanilide (Merck Index, No. 8299); domiphen bromide (Merck Index, No. 3411); cetylpyridinium chloride, (CPC) (Merck Index, No. 2024); tetradecylpyridinium chloride, (TPC); N-tetradecyl-4-ethylpyridinium chloride (TDEPC); octenidine; delmopinol, octapinol, and other piperidino derivatives; nicin preparations; zinc/stannous ion agents; antibiotics such as augmentin, amoxicillin, tetracycline, doxycycline, minocycline, and metronidazole; and peroxides, such as cylium peroxide, hydrogen peroxide, and magnesium monoperthalate and its analogs as described in U.S. Pat. No. 4,670,252; and analogs and salts of the above antimicrobial antiplaque agents. If present, the antimicrobial antiplaque agents may comprise from about 0% to about 6%, preferably from about 0.1% to about 5% by weight of the compositions of the present invention.

Anti-inflammatory agents can also be present in the oral compositions of the present invention. Such agents may include, but are not limited to, non-steroidal anti-inflammatory agents such as ketorolac, flurbiprofen, ibuprofen, naproxen, indomethacin, aspirin, ketoprofen, piroxicam and meclofenamic acid. If present, the anti-inflammatory agents

generally comprise from about 0.001% to about 5% by weight of the compositions of the present invention.

Nutrients can also be present in the oral composition of the present invention, on condition that they are compatible with the SmaA protein or fragment. Such agents may include folate, retinoids (Vitamin A), Vitamin C, Vitamin E and zinc. If present, the nutrients generally comprise from about 0.001% to about 10% by weight of the compositions of the present invention.

Other optional ingredients include a safe and effective amount of a fluoride ion source, which typically is in the form of a water-soluble fluoride compound. This water-soluble fluoride compound is typically present in the compositions of the present invention in an amount sufficient to give a fluoride concentration of from about 0.0025% to about 5.0% by weight, preferably from about 0.005% to about 2.0% by weight. Preferred fluoride sources are sodium fluoride, acidulated phosphate fluoride, and sodium monofluorophosphate. U.S. Pat. No. 3,678,154, issued Jul. 18, 1972 to Widder et al., the disclosure of which is incorporated herein by reference, discloses such salts as well as others.

Compositions of the present invention may also include one or more anticalculus agents, on the condition that they are compatible with the SmaA protein or fragment. Anticalculus agents which may be useful in the compositions of the present invention include diphosphonates such as 1 - azocycloheptane-2,2-diphosphonate AHP) and ethane-1-hydroxy-1,1 - diphosphonate (EHDP), sodium zinc citrate, phosphocitrate, tripolyphosphate, and linear polycarboxylate (LPC); pyrophosphates or polyphosphates such as those disclosed in U.S. Pat. No. 4,590,066 issued to Parran & Sakkab on May 20, 1986 (e.g. tetrasodium pyrophosphate, tetrapotassium pyrophosphate, and dihdrogen disodium pyrophosphate); polyacrylates and other polycarboxylates such as those disclosed in U.S.

Pat. No. 3,429,963 issued to Shedlovsky on Feb. 25, 1969 and U.S. Pat. No. 4,304,766 issued to Chang on Dec. 8, 1981; and U.S. Pat. No. 4,661,341 issued to Benedict & Sunberg on Apr. 28, 1987; polyepoxysuccinates such as those disclosed in U.S. Pat. No. 4,846,650 issued to Benedict, Bush & Sunberg on Jul. 11, 1989; nitrilotriacetic acid and related compounds as disclosed in U.S. Pat. No. 3,678,154 issued to Widder & Briner on Jul. 18, 1972; polyphosphonates as disclosed in U.S. Pat. No. 3,737,533 issued to Francis on Jun. 5, 1973, U.S. Pat. No. 3,988,443 issued to Ploger, Schmidt-Dunker & Gloxhuber on Oct. 26, 1976 and U.S. Pat. No. 4,877,603 issued to Degenhardt & Kozikowski on Oct. 31, 1989; all of these patents are incorporated herein by reference. If present, the anticalculus agents generally comprise from about 0.2% to about 13%, preferably from about 0.4% to about 6% of the compositions of the present invention. Preferred anticalculus agents are pyrophosphate and AHP.

As indicated, compositions of the present invention may be in the form of toothpastes. Components of such toothpastes generally include a dental abrasive (from about 10% to about 50%), a surfactant (from about 0.5% to about 10%), a thickening agent (from about 0.1% to about 5%), a humectant (from about 10% to about 55%), a flavoring agent (from about 0.04% to about 2%), a sweetening agent (from about 0.1% to about 3%), a coloring agent (from about 0.01% to about 0.5%) and water (from about 2% to about 45%). Such toothpastes may also include one or more of an anticaries agent (from about 0.05% to about 0:3% as fluoride ion), an anticalculus agent (from about 0.1% to about 13%), and an antiplaque agent (from about 0.1% to about 5%).

Other compositions of the present invention are mouthwashes and mouth sprays. Components of such mouthwashes and mouth sprays include water (from about 45% to about 95%), ethanol (from about 0% to about 25%), a humectant (from about 0% to about 50%), a surfactant agent

(from about 0.01% to about 7%), an flavoring agent (from about 0.04% to about 2%), a sweetening agent (from about 0.1% to about 3%), and a coloring agent (from about 0.001% to about 0.5%). Such mouthwashes and mouth sprays may also include one or more of an anticaries agent (from about 0.05% to about 0.3% as fluoride ion), an anticalculus agent (from about 0.1% to about 3%), and an antiplaque agent (from about 0.1% to about 5%).

Other compositions of the present invention are dental solutions. Components of such dental solutions generally include water (from about 90% to about 99%), preservative (from about 0.01% to about 0.5%), thickening agent (from about 0% to about 5%), flavoring agent (from about 0.04% to about 2%), sweetening agent (from about 0.1% to about 3%), and surfactant (from 0% to about 5%).

Other preferred compositions may be non-aqueous mouth rinses. Suitable components are disclosed in U.S. Pat. No. 4,312,889 issued Jan. 26, 1982 to Melsheimer, and in U.S. Pat. No. 5,143,720 issued Sept. 1, 1992 to Lopes, both incorporated herein by reference. Alcohol free mouth rinses are also preferred. Suitable compositional components can disclosed in U.S. Pat. No. 4,919,918, issued Apr. 24, 1990 to Cole et al., in U.S. Pat. No. 5,283,056, issued Feb. 1, 1994 to Chung et al., in U.S. Pat. No. 5,284,648, issued Feb. 8, 1994 to White et al., and in PCT Appl. No. 9 401 081, published Jan. 20, 1990.

Other embodiments of the oral compositions herein include lozenges. Suitable lozenge components (e.g. a candy base) are disclosed in U.S. Pat. No. 4,931,473, issued Jun. 5. 1990, to Kelleher et al., and in U.S. Pat. No. 4,139,627, issued Feb. 13, 1979 to Lane et al., and in PCT Appl. No. 9 401 081, of Konopa, published Jan. 20, 1994.

Other compositions include chewing gums. Chewing gum components (e.g. gum base, flavoring and sweetening agents) are disclosed in U.S. Pat. No. 4,083,955, issued Apr. 11, 1978 to Grabenstetter et al.

The pH of the subject compositions and/or its pH in the mouth can be any pH which is safe for the mouth's hard and soft tissues. Such pH's are generally from about 3 to about 10, more preferably from about 4 to about 8.

When the oral composition is a toothpaste, typically from about 0.3 grams to about 15 grams, preferably from about 0.5 grams to about 5 grams, more preferably from about 1 to about 2 grams, of toothpaste is applied to an applicating device e.g., a toothbrush. The applicating device is then contacted with the oral cavity surfaces in a manner such that the oral composition is contacted with tissue of the oral cavity, especially the teeth and gums. The applicating device may be further used to effect an even distribution of the oral composition to the tooth surface, for example by brushing. The application preferably lasts for a period of from about 15 seconds to about 10 minutes, more preferably from about 1 minute to about 2 minutes. Following application, the toothpaste residue is typically removed from the tooth surface by using a liquid acceptable to the oral cavity, typically water, to rinse the oral cavity.

When the oral composition is a mouthwash, typically from about 1 ml. to about 20 ml, preferably from about 2 ml to about 15 ml., most preferably from about 10 ml to about 15 ml, of liquid mouthwash containing the SmaA protein or active fragment is introduced to the oral cavity. The liquid mouthwash is then agitated for from about 10 seconds to about 30 min., preferably from about 15 seconds to about 3 min., more preferably from about 30 seconds to about 2 minutes, within the oral cavity to obtain an improved distribution of the mouthwash over the tissue of the oral cavity.

Following agitation, the mouthwash is typically expectorated from the oral cavity.

Application frequency is preferably from about once daily to about 4 times daily, more preferably from about 3 times weekly to about 3 times daily, more preferably still from about once to about twice daily. The period of such treatment typically ranges from about one day to a lifetime.

In one aspect of the invention, an amylase binding polypeptide sequence representing a fragment or portion of the SmaA protein can be used to competitively inhibit binding of *S. mutans* to the salivary pellicle. Such polypeptides can for example be derived from a recombinant or naturally-derived full SmaA protein, and identification and isolation of the amylase-binding fragment. Alternatively, a DNA sequence encoding an amino acid sequence including the amylase-binding domain of SmaA protein can be utilized in the expression of a recombinant, amylase-binding fragment. It is contemplated that such SmaA fragments will have a molecular weight of at least about 0.5 kd, generally in the range of about 0.5 kd up to about 20 kd. The amylase binding site of the SmaA protein is expected to reside upon the exposed portion of the SmaA protein, and its identification can be achieved by standard methods including for example analysis of the amino acid sequence for the SmaA protein for hydrophilic domains characteristic of exposed domains, deletion analysis, amylase-binding assays, and other techniques well known to those of ordinary skill in the art.

In one form of the invention, the amylase-binding fragment will be of a size rendering it non-immunogenic to the host. As is known in the art, polypeptides having molecular weights less than about 10 kd will usually not elicit an immune response, and thus fragments of less than about 10 kd will desirably be utilized in this feature of the invention. More preferably,

the non-immunogenic fragment will have a molecular weight in the range of about 0.5 kd up to about 5 kd, and especially about 0.5 kd up to about 2 kd.

The present invention also provides recombinantly produced SmaA polypeptides and genetic materials and methods useful in making such polypeptides. The SmaA DNA can, for example, be operably associated with a promoter and incorporated in a suitable vector, e.g. a transformation vector, and used to transform cells *in vitro* or *in vivo*. In one aspect, microorganisms can be transformed using these inventive DNA sequences and caused to express a SmaA polypeptide *in vitro*. For example, using methods well known in the relevant art, microorganisms, including but not limited to bacteria such as *E. coli* cells, may be transformed such that they synthesize inventive proteins in relatively large amounts. Unicellular hosts are selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded on expression by the DNA sequences of this invention to them, their secretion characteristics, their ability to fold proteins correctly, their stability and culturing requirements, and the ease of purification of the products coded on expression by the DNA sequences of this invention. Given these paramaters, skilled practitioners will be able to select a variety of suitable hosts for use in this aspect of the invention. In other aspects, such vectors or other DNA constructs can be used to transform a host animal *in vivo* to express a SmaA polypeptide, for example an antigenic polypeptide in the case of a DNA vaccine.

The present invention is not intended to be limited by the choice of vector or host cell. It should of course be understood that not all vectors and expression control sequences will function equally well to express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences, and hosts without undue experimentation and without departing from the scope of this invention.

Similarly, it will be understood that it is oftentimes convenient or even preferred that recombinant or synthetic production yields a protein modified from its native form, that is, a polypeptide that differs as to its structure from the naturally occurring polypeptide, but which retains the biological activity of the naturally occurring polypeptide. For example, a modified SmaA polypeptide may contain one or more additional amino acids, at one or both ends of the polypeptide chain, may include one or more point mutations, deletions, truncations, or the like. SmaA polypeptides incorporating such modifications while exhibiting the amylase-binding properties of the native SmaA polypeptide are contemplated as being within the present invention.

For the purpose of promoting a further understanding and appreciation of the present invention and its advantages, the following specific Examples are provided. It will be understood that these examples are illustrative, and not limiting, of the present invention.

EXAMPLE 1

PURIFICATION AND CHARACTERIZATION OF SMAA PROTEIN AND USE IN INHIBITING BINDING OF *S. MUTANS* FIMBRIAE

1.1 MATERIALS AND METHODS

A. Bacteria. A *S. mutans* isolate from the saliva of a 7 yr old caries active child (defined as having $\geq$ 5 unrestored surfaces and designated strain A32-2) was used in all experiments and was maintained in 5% CO_2 and 95% air at 37°C overnight in Todd Hewitt broth (Difco Laboratories, Detroit, MI) and passed a minimum number of times. This strain has previously been described to be heavily fimbriated.

B. Fimbrial preparation. A modification (M. Fontana et al, Clin. Diag. Lab. Immun. 2:719-725 (1995); M. Perrone et al., Clin. Diag. Lab. Immun. 4:291-296 (1996)) of the technique of McBride and colleagues (E.J. Morris et al., J. Bacteriol. 169:164-171 (1987)) for isolating fimbriae from *S. sanguis* whole cells, was used for the removal of *S. mutans* fimbriae. The procedure utilized alternating high and low speed centrifugations. *S. mutans* was grown in nine liters of Todd Hewitt broth for 18 h at 37°C in 5% CO_2 and 95% air. Cells were pelleted and washed once gently at 16,274 *x g* at 4°C for 10 min in fimbriae buffer (10 mM phosphate-buffered saline, 1 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride [PMSF]; pH 7.2) and frozen as a pellet at -20°C overnight. PMSF was added to inhibit endogenous proteolytic digestion of fimbrial proteins and $CaCl_2$ was used to reduce fimbrial aggregation. Frozen cells were thawed, suspended in fimbriae buffer and fimbriae were removed by a Waring blender using two one-min cycles at high speed. Following blending, the sample was centrifuged (16,274 *x g*, 4°C, 10 min) to remove intact cells and cell debris and the fimbriae preparation in the supernatant was isolated by ultracentrifugation (110,000 *x g*, 4°C, 2 h). The pellet containing the fimbriae preparation was resuspended in fimbrial buffer and centrifuged (16,274 *x g*, 4°C, 10 min) to remove cellular debris and aggregated fimbriae and the supernatant was divided into aliquots and stored at -80°C. The protein concentration was determined using the Bio-Rad micro-protein assay (Bio-Rad Laboratories, Hercules, CA).

C. Preparation of salivary components and the purified fimbrial protein. Saliva was collected from seven healthy individuals [neither caries free (no decayed, missing or filled surfaces) nor caries active ($\geq$ 5 unrestored surfaces)] and stored at -20°C. Prior to use, the salivas were clarified by centrifugation (2,800 *x g*, 4°C, 10 min) and protein concentrations determined. Saliva samples were diluted to 500 µg of protein/ml in physiological saline for SDS-PAGE or in 0.1 M carbonate/bicarbonate buffer (pH 9.6) for ELISA. In order to separate

salivary protein fractions, preparative gel electrophoresis (Prep cell model 491; Bio-Rad) was utilized. The resolving and stacking gels were composed of 10% and 3% acrylamide (National Diagnostics, Atlanta, GA), respectively. A clarified whole saliva sample (2 ml) was added to an equal volume of SDS-PAGE sample buffer, boiled for 7 min and placed on a 6 cm column and subjected to 12 W of continuous power. The protein of interest eluted after approximately 8 h of electrophoresis and was previously determined by immunoblots of whole saliva to have a molecular weight of approximately 52 kDa. The proteins were collected and analyzed for molecular weight and purity by gel electrophoresis after staining with coomassie brilliant blue. The fractions of interest were pooled and passed through an affinity column that removes SDS (Extracti-Gel, Pierce, Rockford, IL) and stored at -80°C. Purification of the immunodominant 65 kDa fimbrial protein identified earlier (M. Fontana et al, Clin. Diag. Lab. Immun. 2:719-725 (1995); M. Perrone et al., Clin. Diag. Lab. Immun. 4:291-296 (1996)) was also accomplished by preparative gel electrophoresis using an identical method. Rat antisera to the enriched A32-2 fimbrial preparation and to the 65 kDa fimbrial protein were obtained from eight animals each immunized with 5 μg protein/ml incorporated into the RIBI adjuvant system (RIBI ImmunoChem Research, Inc., Hamilton, MT). Preparations were injected with 0.2 ml s.c. in each of two sites and 0.1 ml i.p. twice 21 days apart and blood collected 7 days after the last injection. The blood was allowed to clot and serum was obtained and frozen at –20°C until used.

D. ELISA assay for binding of fimbriae and fimbrial protein to whole saliva and salivary components. Whole saliva (undiluted and 1:2 and 1:10 dilutions), purified 52 kDa salivary protein (65.0 μg/ml) and human salivary α-amylase (10.0 μg/ml; Type IX A, Sigma Chemical Co., St. Louis, MO) were assayed to determine the ability of fimbriae and the fimbrial protein to bind to salivary components. Polystyrene 96 well microtitre plates (Flow Laboratories, Inc., McLean, VA) were coated (100 μl) with the

salivary components or whole saliva (diluted in 0.1 M carbonate/bicarbonate buffer; pH 9.6) and incubated for 3 h at 37°C or overnight at 4°C. The unbound salivary components were removed by washing three times with normal saline containing 0.05% Tween 20 (Tween-saline). A solution of 1% bovine serum albumin (BSA; Sigma) in carbonate/bicarbonate buffer was added (200 µl) to block any unbound sites and incubated for 1 h at 25°C. Following washing, 100 µl of the A32-2 fimbrial preparation (33.0 µg/ml in saline), purified 65 kDa fimbrial protein (1 µg/ml) or Tween-saline (no fimbriae control) was added and incubated for 3 h at 37°C and washed 3 times. Rat antibody to the A32-2 fimbriae preparation or rat anti-65 kDa fimbrial protein (both diluted 1:4000 in Tween-saline) was added (100 µl) and incubated for 3 h at 37°C. After washing, goat antibody to rat IgG (Fc specific) conjugated to horseradish peroxidase (Sigma) was added (100 µl; 1:8000 dilution) and incubated for 3 h at 37°C. After a final wash step, the substrate (10 mg of orthophenylenediamine dihydrochloride and 14 µl of 30% H_2O_2 in 20 ml of 0.5 M citrate buffer [pH 5.0]) was added (100 µl) and developed for 30 min and read at 490 nm using a microplate spectrophotometer (Molecular Devices Corp., Menlo Park, CA). In addition, a modification of the ELISA assay described above was used to determine the efficacy of the purified 52 kDa salivary protein in inhibiting the binding of *S. mutans* A32-2 fimbriae to a 1:10 dilution of whole saliva. Mixtures of the *S. mutans* fimbriae preparation (33.0 µg/ml) and serially diluted 52 kDa salivary protein (0.5-65.0 µg/ml) were incubated for 30 min at 37°C and used in place of the untreated fimbriae preparation. Controls included whole saliva and BSA.

E. Immunoblot analysis for binding of fimbriae to salivary components and amylase detection. In order to determine which components in whole saliva bound *S. mutans* fimbriae, reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used (U.K. Laemmli, Nature 227:680-685 (1970)). The resolving and stacking

gels were composed of 10% and 3% acrylamide, respectively. Saliva samples (50 µl samples in saline) were boiled for 7 min and electrophoresed using a mini-gel electrophoresis apparatus (Mini-Protean II; Bio-Rad) for 60 min at a voltage of 150V. After electrophoresis, separated proteins on the gel were transferred to nitrocellulose paper (Bio-Rad) overnight at 4°C at a constant voltage of 30V in a mini-transblot electrophoretic transfer cell (31; Bio-Rad). The nitrocellulose paper was blocked in a solution of 1% defatted milk (Carnation instant milk, Carnation Company, Los Angeles, CA) diluted in washing buffer (0.9% NaCl containing 0.5% Tween-20; WBT) for 2 h at 25°C. The nitrocellulose paper was washed with WBT 3 times for 10 min each, the *S. mutans* fimbrial preparation (33 µg/ml; 2 ml) in WBT was added and incubated for 1 h at 25°C. The membrane was washed to remove unbound protein and incubated with rat antibody to A32-2 fimbriae (diluted 1:500 in WBT) for 1 h at room temperature. Goat antibody to rat IgG (Fc specific)-alkaline phosphatase conjugate (1:1000 in WBT; Sigma; 100 µl) was added and incubated for 1 h. Binding of the antibody was detected by addition of alkaline phosphatase substrate (p-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate; Bio-Rad) dissolved in 100 mM Tris HCl (pH 9.5). In order to determine whether the 52 kDa salivary protein was amylase, the isolated salivary protein (65.0 µg/ml), commercial purified amylase (10.0 µg/ml) and undiluted whole saliva were electrophoresed in SDS-PAGE, transferred to nitrocellulose and probed with rabbit anti-human α amylase (Sigma) followed by alkaline phosphatase-labeled goat anti-rabbit IgG (Sigma) and substrate similar to the method described above.

F. Statistical analysis. The data were reduced by computing the means and standards errors of the mean of the absorbances of triplicate determinations per sample. The data were analyzed by Student's t test and significant differences were defined as $p \leq 0.05$.

1.2 RESULTS

A. Fimbriae binding assays. ELISA and immunoblotting were used to establish binding of the *S. mutans* fimbriae preparation to saliva-coated surfaces. An ELISA was performed to determine if a *S. mutans* fimbriae preparation from strain A32-2 bound to human whole saliva. Fimbriae from *S. mutans* A32-2, a strain isolated from a caries active subject, demonstrated significant binding as compared with the corresponding Tween-saline control (no fimbriae; Fig. 1). The binding of fimbrial components to saliva was reduced when either the saliva or fimbriae was diluted. These data provided the first indication that *S. mutans* fimbriae had binding activity with saliva-coated surfaces. BSA-coated wells did not bind fimbriae (data not shown).

B. Immunoblot analysis of human whole saliva probed with *S. mutans* A32-2 fimbriae. The binding activity of the *S. mutans* A32-2 fimbriae preparation to separated salivary proteins was analyzed by immunoblotting. Human whole saliva samples were collected from seven healthy subjects. Each saliva sample was electrophoresed, transferred to nitrocellulose paper and probed with the *S. mutans* fimbriae preparation. Fimbriae from the A32-2 strain bound strongly to a 52 kDa salivary protein in all 7 saliva samples (Fig. 2). Controls with no fimbriae did not reveal any bands.

C. Isolation of a 52 kDa salivary protein with *S. mutans* fimbriae-binding activity. In order to better understand the interaction between the 52 kDa salivary protein and *S. mutans* fimbriae, the salivary protein was isolated by preparative gel electrophoresis. Following elution, the fractions were analyzed by gel electrophoresis and fractions were identified that contained only one band (Fig. 3).

D. ELISA assay for binding of *S. mutans* fimbriae and purified 65 kDa fimbrial protein to isolated salivary protein, amylase and whole saliva. In order to ascertain that both the salivary protein and amylase have fimbrial-binding characteristics, an ELISA was employed to measure binding. Amylase was chosen because the molecular weight was similar to 52 kDa and several oral streptococci have demonstrated the ability to bind to amylase. In this assay, amylase (10.0 µg/ml) had significantly greater fimbrial-binding activity than the no fimbriae Tween-saline control (Fig. 4). Amylase also had a significantly greater absorbance compared to diluted whole saliva (0.5 µg/ml). The isolated salivary protein (65.0 µg/ml) had a lower absorbance than either amylase or whole saliva, but the absorbance was significantly higher than the no fimbriae control. Purified 65 kDa fimbrial protein bound similarly to amylase (O.D. 0.250 $\pm$ 0.026) as to a 1:2 dilution of saliva (O.D. 0.260 $\pm$ 0.030), but not to a Tween-saline negative control (0.070 $\pm$ 0.012).

E. Inhibition of binding of *S. mutans* fimbriae to whole saliva-coated surfaces. In binding assays, an important feature is the ability to inhibit the interaction. The ability to inhibit binding suggests that the interaction is specific. In this system, the purified salivary protein was incubated with the fimbriae preparation from *S. mutans* A32-2. Following incubation with the salivary protein, the mixture was added to whole saliva. The data indicated an inverse relationship between the concentration of the salivary protein and the extent of binding of the *S. mutans* fimbriae preparation to whole saliva (Fig. 5). Whole saliva and BSA controls indicated complete and no inhibition, respectively.

F. Immunoblot analysis of the purified salivary protein probed with anti-human α-amylase antibody. The purified salivary protein, human amylase and whole saliva were assayed for reactivity with rabbit antibody to human α-amylase. The results indicated that all three salivary

preparations contained components that were recognized by the anti-amylase antibody (Fig. 6).

EXAMPLE 2

CLONING AND EXPRESSION OF SmaA PROTEIN

2.1 Cloning of saliva- and amylase-binding protein. Genomic DNA from a heavily fimbriated strain of *S. mutans* was digested by *Sau*3A partial digestion and cloned into a *BamH*I site of a λ gt11 universal expression vector (EMBL3; Promega Corp., Madison, WI). *E.* coli LE392 was transfected with λ and viral plaques lifted onto nitrocellulose paper and probed with monospecific antisera to the 65 kDa fimbrial protein, and polyclonal antisera to crude fimbriae or to *S. mutans* whole cells. One clone reacted strongly with the anti-65 kDa antisera and less strongly with the anti-crude fimbriae or anti-*S.* mutans whole cells and was repropagated and reprobed with anti-65 kDa fimbrial antisera (Fig. 7). Every plaque produced a positive spot on the plaque lift. Since there were *Sal*I sites flanking the *BamH*I site (1 base away) in the EMBL3 arm of λ, fimbrial genes, including that encoding for the 65 kDa protein, were identified in λ phage as a 9.5 kb *Sal*I insert (Fig. 8; lane 2). Restriction enzyme digestion of the purified 9.5 kb *S. mutans* insert from transfected λ plaques were conducted. The 9.5 kb insert had apparent *Sma*I (6.1 and 3.5 kb fragments) and *Hind*III (5.0 and 4.5 kb fragments) sites and did not have *EcoR*I or *Sst*I sites. λ phage lysate from transfected, but not control, preparations contained expressed *S. mutans* fimbrial components that bound to both saliva and amylase (Fig. 9, Table 1). These results indicate that the 65 kDa SmaA protein component of *S. mutans* fimbriae is carried on the 9.5 kb fragment and participates in adherence of the bacteria to the salivary pellicle, and that amylase serves as an adhesion factor.

TABLE 1
Slot Blot map and absorbance data

Columns	Row 1	Row 2	Row 3	Row 4	Row 5	Row 6
A	Blank 0.145	Sal-10-Fim No Ab 0.317	Am25 Fim FimAb 0.171	Am50 Fim FimAb 0.312	Sal-20 Fim FmAb 0.381	Sal-10 Fim FimAb 0.354
B	Blank 0.126	Sal-10-Fim NoAb 0.327	Am25 Fim FimAb 0.193	Am50 Fim FimAb 0.337	Sal-20 Fim FimAb 0.376	Sal-10 Fim FimAb 0.344
C	Blank 0.099	Sal-20 -Fim NoAb 0.205	Am25 Ph-3 FimAb 0.061	Am50 Ph-3 FimAb 0.090	Sal-20 Ph-3 FimAb 0.364	Sal-10 Ph-3 FimAb 0.381
D	Blank 0.060	Sal-20 -Fim NoAb 0.239	Am25 Ph-3 FimAb 0.056	Am50 Ph-3 FimAb 0.085	Sal-20 Ph-3 FimAb 0.388	Sal-10 Ph-3 FimAb 0.391
E	Blank 0.060	Am50 -Fim NoAb 0.061	Am25 Ph-4 FimAb 0.073	Am50 Ph-4 FimAb 0.075	Sal-20 Ph-4 FimAb 0.366	Sal-10 Ph-4 FimAb 0.396
F	Blank 0.060	Am50 -Fim NoAb 0.053	Am25 Ph-4 FimAb 0.059	Am50 Ph-4 FimAb 0.073	Sal-20 Ph-4 FimAb 0.420	Sal-10 Ph-4 FimAb 0.396
G	Blank 0.067	Am25 -Fim NoAb 0.046	Am25 Ph-5 FimAb 0.054	Am50 Ph-5 FimAb 0.066	Sal-20 Ph-5 FimAb 0.420	Sal-10 Ph-5 FimAb 0.366
H	Blank 0.040	Am25 -Fim NoAb 0.048	Am25 Ph-5 FimAb 0.054	Am50 Ph-5 FimAb 0.083	Sal-20 Ph-5 FimAb 0.415	Sal-10 Ph-5 FimAb 0.398

EXAMPLE 3

ISOLATION OF AMYLASE BINDING SmaA FRAGMENT

A purified preparation of SmaA protein is subjected to tryptic digestion with 10 µg/ml for 30 minutes at 37°C. The sample is separated by SDS-PAGE using a 10-20% gradient gel. The separated peptides are transferred from the gel to immunoblotting paper and probed with 10 µg/ml human salivary alpha-amylase (Sigma Chemical Co.) for 1 hour at room temperature. After washing the blot, rabbit anti-human amylase antibody is added and incubated for 1 hour, washed and alkaline phosphatase-labeled goat anti-rabbit IgG antibody added and incubated for 1 hour. After washing, NBT/BCIP substrate is added for color development. The peptide band or bands that bind amylase will have a color change and their molecular weight is determined. After establishing the molecular weight of the amylase binding peptide, the peptide is purified from a tryptic digest by preparative electrophoresis and can be used in functionally blocking *S. mutans* adherence to the salivary pellicle.

EXAMPLE 4

SUBCLONING AND CHARACTERIZATION OF SmaA DNA

4.1 Subcloning of the 65 kDa fimbrial protein gene. In order to obtain the λ insert carrying the 9.5 kb *S. mutans Sal*I fragment, anti-65 and anti-A32-2 fimbrial preparation-positive plaques are reisolated (3 times total) on fresh *E. coli* LE392 cells (with maltose and $MgSO_4$) to get 100% positive λ phage with the desired fimbrial gene. The λ phage DNA is isolated from positive cultures and *Sal*I-digested to obtain the 9.5 kb insert. This insert is then be digested with a number of enzymes. A panel of restriction enzymes (single and double digestions) is used to cut DNA. All bands <9 kb are cut out, the DNA eluted and ligated into pDL289, pDL290 or other similar vector and transformed into *E. coli* on kanamycin-containing

LB agar plates. Plasmids are isolated from selected kanamycin-resistant colonies by a rapid streptococcal plasmid screening procedure (D.G. Anderson et al., Appl. Environ. Microbiol., 46:549-552 (1983)) and colonies screened for fimbrial protein by probing colony lifts with antisera as described below. The kanamycin-resistant colonies expressing 65 kDa fimbrial protein and carrying the smallest piece of *S. mutans* DNA is of primary interest for restriction enzyme mapping and sequencing.

Briefly, the A32-2 is first examined for transformability by natural transformation with pDL278 carrying a kanamycin resistance marker. *E. coli* DH5α cells are transformed by the calcium chloride method as described by Ausubel et al. (Current Protocols in Molecular Biology, 1987). *S. mutans* is transformed or is electroporated by the method of Caparon and Scott (Methods in Enzymol., 204:556-586 (1991)). If neither natural transformation or electroporation with A32-2 is successful, other heavily fimbriated strains from CA subjects (M. Perrone et al., Clin. Diagn. Lab. Immunol., 4:291-296 (1997)) are assayed using natural transformation and electroporation.

Small scale isolation of *E. coli* plasmid DNA is performed as described by Birnboim and Doly (Nuclei Acids Res., 7:1513-1523 (1979)). Large scale isolation is done by Birnboim and Doly (*id.*) as described by Sambrook et al. (Molecular Cloning: A Laboratory Manual. 2nd Edition). Screening of *S. mutans* plasmid DNA is by a modification (N.D. Buckley et al., Genetics of Streptococci, Enterocci and Lactococci, Vol. 85, pp. 399-410 (1995); N.D. Buckley et al., J. Bacteriol., 177:5028-5034 (1995)) of a procedure described by Anderson and McKay (Appl. Environ. Microbiol., 46:549-552 (1983)). Briefly, mutanolysin (12 U/ml; Sigma) is used to lyse the cells in 1.5 ml of culture.

The 9.5 kb and other smaller restriction endonuclease fragments are subcloned into either pDL289 or pDL290 in both orientations and examined

for 65 kDa fimbrial protein expression by colony blotting as described below. If protein is expressed in either orientation, the promoter must be on the cloned fragment, but if protein is expressed in only one orientation when the *lac*Z promoter is induced with PITG, the fimbrial protein gene must be using the *lac*Z promoter and may be part of an operon on the *S. mutans* chromosome. If protein is expressed in both directions, then exonuclease III is used to digest the fragment to different sizes in both directions as described by Henikoff (Gene, 28:351-359 (1984)) and Reznikoff et al. (Methods in Enzymology, 217:312-322 (1993)). The nested deletions are used to subclone back into the plasmid and examined for protein expression. In addition, the overlapping nested deletions are saved for sequencing. The overlapping nature of the nested deletions will allow the sequencing to be more accurate and efficacious. Alternatively if the insert can not be subcloned into pDL289 or pDL290, the 9.5 kb or other inserts are subcloned into pET which carries a *lac*Z promoter and the cells are induced with IPTG.

Natural transformation of *S. mutans* is conducted by incubating 10 ml of overnight culture in BHI without glucose and adding to 200 ml of BHI broth containing 10 mM glucose. Five ml of that are transferred to a screw top tube and incubated until the $O.D_{0.660nm}$ is approximately 0.5. The culture is chilled on ice and the cells pelleted by centrifugation. The cells are resuspended in 10 ml of cold BHI and 30% glycerol and aliquoted into 150 µl aliquots and frozen in a dry ice-ethanol bath and the frozen competent cells stored at -85ºC. Later 0.1 ml of the thawed cells are transferred to 10 ml of BHI and then 0.1 ml of that transferred to 10 ml of BHI containing 10 mM glucose and 10% heat inactivated horse serum. The cells are incubated for exactly 4 h and 30 min (previously optimized time interval for each lot of serum) at 37ºC in an anaerobic GasPak jar. DNA to be transformed is added (10 µl) to 100 µl of competent cells and incubated an additional 4 h in the GasPak jar. The cells are spread on selective plates containing either kanamycin (35 or 250 µg/ml for *E. coli* or

streptococci, respectively) or spectinomycin (50 or 250 µg/ml for *E. coli* or streptococci, respectively) if using the spectinomycin gene from *Enterococcus facaelis* and incubated for 48 h.

To demonstrate colonies expressing fimbrial proteins, colonies are grown until approximately 0.1 - 0.2 mm in size. Colonies are transferred to a dry sterile nitrocellulose filters (Millipore HAWP or equivalent). The filter is transferred, colony side up, to a prewarmed agar plate containing antibiotic and incubated for 2-4 h at 37ºC to allow further growth of cells. The filters are placed on a damp paper towel and then hung over fresh chloroform for 30-40 min to lyse the cells. The filters are blocked in tris buffered saline containing 5% dry skim milk for 1 h. The filters are incubated with the rat anti-65 kDa fimbrial protein antisera, washed and incubated in alkaline phosphatase labeled anti-rat IgG, washed, and substrate added for color development. Crude cell lysates from positive strains are prepared and used for saliva- and amylase-binding studies.

4.2 Restriction endonuclease digestion of fimbrial gene(s). For routine digestions, the methods described by the enzyme manufacturer are followed. For restriction enzyme mapping of the 65 kDa fimbrial gene, a panel of enzymes (Gibco BRL Life Technologies, Frederick, MD) is used in single and double digestions.

4.3 Sequencing the *S. mutans* fimbrial gene(s). The nucleotide sequences of selected nested sequence deletion derivatives are determined using the method of Sanger et al. (Proc. Natl. Acad. Sci. USA, 74:5463-5467 (1977)).

EXAMPLE 5

ANALYSIS OF SmaA POLYPEPTIDES FOR INHIBITION OF DENTAL CARIES FORMATION

SmaA polypeptides (including fragments) are screened for their ability to inhibit dental caries formation as follows.

5.1 Analysis of colonization and cariogenicity of knockout mutants using an *in vitro* caries model. For each study, four groups of 12 human teeth specimens per group are treated for a 4-day test period in an *in vitro* microbial artificial-mouth caries model. The groups will differ from each other in the presence (A1, A2, and A3) or absence (B1: negative control) of *S. mutans* (serotype *c*), and in the concentration of SmaA (10-500 µg/ml) used. Enamel specimens (3 mm in diameter) are drilled from extracted, sound, human, lower permanent incisors which had been obtained from oral surgeons and sterilized by soaking in 3% buffered (neutral) formalin since the time of extraction. Each specimen is mounted on a polyacrylic rod using methyl methacrylate resin. The specimens are ground using 600-grade silicon carbide paper to remove approximately 50 µm of the surface and then polished to a high luster with Gamma Alumina (0.05 µm) using standard methods. The specimens are then randomly assigned to test groups, each group initially composed of 14 specimens. All specimens are sterilized with ethylene oxide gas. Two specimens from each group are randomly chosen before treatment and examined to obtain baseline confocal microscopy data. The 12 specimens that remained in each group are secured in caries-forming vessels by gluing the ends of their plexiglass rods to a round plexiglass base that fit in the bottom of the vessels. Trypticase soy broth without dextrose (Difco Laboratories; Detroit, Mich.) (TSBS) is used as the bacterial nutrient broth. For each caries-forming vessel there is one 1 L bottle of TSBS that dispensed the media (0.7 ml/min) at three different times each day, for 30 min each, for a total of 63

ml/day by means of a peristaltic pump (Wiz peristaltic pump; ISCO, Inc., Lincoln, Neb). A mineral wash (MW) solution (pH 7.0) is used to mimic the action of saliva. One liter of MW contained: potassium chloride (624.6 mg); sodium chloride (866.6 mg); dipotassium hydrogen phosphate (33.8 mg); magnesium chloride (59.6 mg); and calcium chloride dihydrate (166.6 mg). Twenty-liter polypropylene bottles (Fisher Scientific; Pittsburgh, PA) are used to store the MW. There is one bottle for each of two groups. Each bottle dispenses approximately 882 ml of MW/day (0.7 ml/min) to each caries-forming vessel, intermittently over a period of 21 h, during the periods with no TSBS flow, by a peristaltic pump. Three times a day, prior to each TSBS cycle, 1 ml of the appropriate SmaA solution is administered to each group by injection, followed by flushing with 5 ml of sterile saline (8.78 g NaCl/L deionized water). The antibody solutions are allowed to mix with fluid (MW) in the caries-vessels by stirring for 30 min before cycling is resumed. All of the media and model components are autoclaved at 121°C for 20 min prior to initiation of each experiment. Each group of 12 specimens is placed in a caries-forming vessel (125-ml Pyrex brand slow speed stirring vessel; Fisher). All caries-forming vessels are placed on an electric stirrer inside an incubator at 37°C under aerobic conditions. Each caries vessel has three inlets, one for TSBS, one for MW, and one for injection of the antibody; and one outlet for drainage tubing. The drainage tubing ended flush in a drainage container, which is also placed inside the incubator. Drainage of fluid from each caries vessel is maintained at 0.7 ml/min by a peristaltic pump (Wiz, ISCO). Each specimen in groups A1, A2, and A3 is inoculated by micropipette with 20 µl of washed, overnight (16 h), stationary-phase cells of *S. mutans S. mutans* A32-2 (serotype *c*), resuspended in TSBS to an optical density of 0.5 OD at 540 nm. Group B1, the negative control, is inoculated with TSBS only. Prior to inoculation, filter (0.2 µm)-sterilized SmaA is placed on the surface of the uncoated or amylase-coated (200 µg/ml) enamel specimens (20 µl) and incubated for 1 h at 37°C. Sterile saline (8.78 g NaCl/L of deionized wter) is used instead

of SmaA in group A1. Following inoculation with 20 µl of bacteria the specimens are incubated for two hours at 37°C to allow the bacteria to implant on the teeth. Each group of specimens is then placed in a separate caries forming-vessel and attached to the MW, TSBS/TSB and drainage container bottles. The following parameters are measured in the supply and drainage containers fluid at the beginning and at the end of the 4-day test periods to monitor the absence of contamination and the viability of the inoculum: 1) pH; 2) *S. mutans* viability (by plating on mitis salivarius agar, Difco; supplemented with 20 % sucrose and 200 IU/L bacitracin); and 3) bacterial contamination (by plating on trypticase soy agar, Difco). Plates are incubated at 37°C in 5 % CO_2 - 95 % air for 3 days. In order to quantitate the bacteria adhered to the teeth, at the end of the test periods teeth from each group are randomly selected and placed individually in 5 ml of sterile saline. Each specimen is then vortexed (20 sec) and sonicated (20 sec) until all visible dental plaque is displaced from the surface of the tooth. All samples are then plated on mitis salivarius and trypticase soy agar. Following termination of each study, caries activity is assessed using standard procedures (Fontana M., A. J. Dunipace, R. L. Gregory, T. W. Noblitt, Y. Li, K. K. Park, G. K. Stookey. 1996. An *in-vitro* microbial model for studying secondary caries formation. Caries Res. 30:112-118).

5.2. Analysis of inhibition of caries formation in a rat caries model. The ability of SmaA protein and fragments to inhibit colonization and caries lesion formation by *S. mutans* is assessed using a standard rat caries model. Briefly, thirty conventional rats (Harland Sprague Dawley) are used per group. Each SmaA polypeptide is assigned to a separate group. At 15 days of age, each rat is challenged with wild type A32-2 on four days (15 and 18-20 days of age). One rat group serves as a positive control (no administration of SmaA polypeptide), and another control group is not infected. Other groups are administered the SmaA polypeptide in their drinking water at levels varying from 10-500 µg/ml. All animals are fed a

standard rat chow diet. Saliva and serum samples are collected at sacrifice (12 weeks after infection; 104 days of age) to determine salivary IgA and serum IgG antibody levels to *S. mutans* A32-2 whole cells, and SmaA polypeptide using general methods as described earlier (M. Fontana et al., Clin. Diagn. Lab. Immunol., 2:719-725 (1995)). At termination, mandibles are assessed for adhered *S. mutans* levels, and then scored for caries. The animals are euthanized during blood collection by intracardiac puncture. The hemijaws (maxilla, mandible) are surgically removed, and the lower right jaw aseptically freed of all soft tissue using a sterile scalpel. This hemijaw (lower right) is used for bacterial counts, and later returned for pressure cooking (10 PSI for 12 min) with the other three hemijaws (lower left, upper right and upper left) for caries scoring. Pressure cooking is done to facilitate soft tissue removal prior to caries scoring. One hemijaw quadrant (right-mandibular) is placed in a tube containing 3 ml of sterile saline (8.77 g NaCl/L of dH_2O), and plaque disrupted from the molar surfaces by vortexing for 20 sec, followed by sonication for 20 sec at a setting of 20 (50 Sonic Dismembrator, Fisher), and finally vortexing again for 20 sec. The number of *S. mutans* cells in the plaque samples is determined by culturing known dilutions (undiluted and 1:10; double plated) of samples, using a Spiral Plater (Spiral Systems) on mitis salivarius-rifampicin and fusidic plates, and incubated at 37ºC. All hemijaws (four quadrants) are stained overnight with a murexide (Sigma) solution (0.3 g of murexide; 300 ml of dH_2O and 700 ml of ethanol) for caries scoring. The jaws are rinsed and allowed to dry. The jaws are examined for smooth surface caries, sectioned, and then microscopically examined for sulcal and interproximal caries using the Keyes method (P.H. Keyes, J. Dent. Res., 37:1088-1099 (1958)).

While various preferred embodiments of the invention have been described in detail above, the same is to be considered illustrative in nature. All modifications and additions as would occur to one of ordinary skill in the field to which this invention pertains are contemplated as being a

part of this invention and are desired to be protected. In addition, all publications cited herein are indicative of the level of ordinary skill in the art, and are hereby incorporated by reference in their entirety as if each had been individually incorporated by reference where cited and fully set forth.